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Isolation and Characterization of *o*-Diphenol-*O*-Methyltransferase cDNA Clone in Hot Pepper (*Capsicum annuum* L.)

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A cDNA clone, *CaOMT1* encoding an *o*-diphenol-*O*-methyltransferase (OMT), which is involved in capsaicin biosynthesis, was isolated by screening of a cDNA library prepared from the mRNA of pepper (*Capsicum annuum* L.) pericarp. Nucleotide sequence analysis of *CaOMT1* revealed that it had an open reading frame of 1080 bp which encodes a polypeptide with a predicted molecular weight of 39,430 D, corresponding well with the size of the known OMT's of tobacco, poplar, aspen, alfalfa, and cabbage. It also had five conserved boxes which appear in all known OMT's. The nucleotide sequence of *CaOMT1* had 89~74% identity with the OMT cDNA's of tobacco, aspen, alfalfa, and poplar, but a relatively lower identity of 59% with the OMT cDNA of maize. Amino acid sequence analysis also revealed that CaOMT1 has high identity with the known OMT's which have a substrate of *o*-diphenolic compounds, especially 5-hydroxyferulic acid and caffeic acid. It supports *CaOMT1* which encodes an OMT. Southern blot analysis suggested that *CaOMT1* might exist in the form of multiple copies in the pepper genome. *CaOMT1* is expressed preferentially in pepper fruit and its expression levels increased during pepper fruit development, but decreased during fruit ripening, suggesting that the *CaOMT1* gene is fruit development-related. *CaOMT1* is the first reported cDNA clone for enzymes related to the phenlypropanoid pathway in pepper.

Keywords: hot pepper (*Capsicum annuum* L.), *o*-diphenol-*O*-methyltransferase, capsaicin, fruit development, phenylpropanoid pathway

Capsaicin (8-methyl-N-vanillyl-6-nonenamide; Bennett and Kirby, 1968) is the major pungent component of hot pepper fruit (*Capsicum annuum* L.) and is used as a spice for hot taste. Capsaicin is synthesized mainly in the placenta of the pepper fruit (Iwai *et al.*, 1979; Suzuki *et al.*, 1981; Fujiwake *et al.*, 1982) and accumulates in the vacuoles of epidermal cells of the placenta (Fujiwake *et al.*, 1980; Suzuki *et al.*, 1980). The accumulation of capsaicin is known to occur over a relatively short period during the latter stages of fruit development (Iwai *et al.*, 1979). Capsaicin is an amide derivative of vanillylamine and *trans*-8-methyl-6-nonenoic acid (Leete and Louden, 1968; Bennett and Kirby, 1968), but the biosynthesis is not fully elucidated. As far as is known, capsaicin-intermediates are made through the phenylpropanoid pathway (Fujiwake *et al.*, 1980). The intermediates can also be used for biosynthesis of monolignols, precursors of lignins, therefore biosynthesis of capsaicin and that of lignin share a common biosynthetic pathway (Sukrasno and Yeoman, 1993). Phenlyalanine ammonia-lyase (PAL), the enzyme of the first step for capsaicin biosynthesis, catalyzes L-phenylalanine into cinnamic acid, which is subsequently converted into *p*-coumaric acid, caffeic acid, and ferulic acid through actions of cinnamic acid-4-hydroxylase (C4H), *p*-coumaric acid-3-hydroxylase (C3H), and *o*-diphenol-*O*-methyltransferase (OMT; EC 2.1.1.6), respectively. The enzymes which are involved in the transformation of ferulic acid into vanillin and vanillin into vanillylamine are unknown. The final step is completed by capsaicinoid synthetase through the enzymatic condensation of vanillylamine and *trans*-8-methyl-6-nonenoic acid.

Generally, the activities of the enzymes, PAL, C4H, C3H, OMT, and CS, participating in capsaicin biosynthesis increase along with the development of the fruit and the growth of callus derived from the hypocotyl of hot pepper (Ochoa-Alejo and Gómez-Peralta, 1993). As for OMT and CS, the activities in

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callus are much lower than those in the fruit. This phenomenon is thought to be the result of the lower expression of OMT and CS in callus (Ochoa-Alejo and Gómez-Peralta, 1993). Therefore, OMT and CS might be the key enzymes for regulation of capsaicin biosynthesis in pepper fruit.

OMT methylates *o*-diphenol compounds such as caffeic acid and 5-hydroferulic acid using *S*-adenosyl-L-methionine as a methyl donor (Collendavelloo *et al.*, 1981). While in gymnosperms, caffeic acid is a main substrate of OMT, in angiosperms, OMT is a bispecific catalyst having substrates of caffeic acid and 5-hydroxyferulic acid, which are converted into ferulic acid and sinapic acid through *O*-methylation by OMT, respectively (Higuchi, 1990). Ferulic acid and sinapic acid are phenolic derivatives for lignin biosynthesis, and ferulic acid may also follow the capsaicin biosynthesis pathway.

Recently, several OMT cDNAs were isolated from aspen (Bugos *et al.*, 1991), alfalfa (Gowri *et al.*, 1991), tobacco (Jaeck *et al.*, 1993; Pellegrini *et al.*, 1993), poplar (Dumas *et al.*, 1992), cider tree (Poeydomenge *et al.*, 1994), *Zinnia elegans* (Ye and Varner, 1995), and OMT genomic genes were isolated from *Zea mays* (Collazo *et al.*, 1992) and poplar (Tsai *et al.*, 1995). However, neither proteins nor the cDNA of CS has been isolated yet.

In this study, we present the basis of molecular studies on the regulation of capsaicin biosynthesis and its pathway.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) and Partial cDNA Cloning

PCR was carried out in a total volume of 50 μL containing 1×10^8 phage particles of hot pepper (*Capsicum annuum* L.) pericarp cDNA library, 50 pmole of OMT-1 primer [ATCGAATTCA(A/G) GTICTIATGGA(A/G)AG(T/C)TGG], 50 pmole of OMT-2 primer [ACTGAATTCTIATIAC(C/T) TTICC(A/G)TTIGCIGG], 2 mM $MgCl_2$, 0.25 mM dNTP, 2.5 units of *Taq* polymerase (Takara), and a reaction buffer supplied by the manufacturer. PCR was performed in a thermal cycler (Pharmacia) for 35 cycles with each cycle consisting of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The amplified products were digested with *Eco*RI, and then electrophoresed in 0.8% agarose gel. The PCR product corresponding to approximately 600 bp was eluted with a JETSORB Kit (GENOMED), and then ligated into pGEM-7Zf(+). The resulting plasmid was designated as pOMT600.

Screening of cDNA Library

A total of 5×10^5 plaques of the hot pepper pericarp cDNA library were plated at a density of 1×10^5 plaques per 150 mm petridish and transferred to Hybond-N+ filters (Amersham) and screened using pOMT600 as a probe. The probe was labeled with [α-^{32}P]dCTP (3000 Ci/mM, Amersham) by the Prime-a-Gene system (Promega). The filters were prehybridized for 3 h at 60°C in 6×SSC, 5×Denhardt's solution, 0.5% SDS, and 100 μg/mL salmon sperm DNA. Hybridization was performed for 16~20 h at 60°C in prehybridization solution with 3×10^6 cpm/mL labeled probes. The filters were washed once in 2×SSC, 0.1% SDS for 10 min, once in 1×SSC, 0.1% SDS for 10 min, once in 0.5×SSC, 0.1% SDS for 10 min, and once in 0.2×SSC, 0.1% SDS for 10 min, at 60°C.

Sequence Analysis

cDNA inserts of the isolated phage clones were recovered by *in vivo* excision from the Uni-ZAP™ XR vector following the manufacturer's instructions. The double-strands of the cDNA insert were sequenced according to the manufacturer's instructions (USB Sequenase ver. 2.0 Kit). For generation of smaller DNA fragments for sequencing, a deletion kit for kilo-sequencing (Takara) was used. DNASIS and PROSIS programs (Hitachi), and the Clustal multiple alignment program (Higgins *et al.*, 1992) were used in nucleotide and amino acid sequence analyses. Nucleotide and amino acid sequence similarities were analyzed through the BLAST Network service of the National Center for Biotechnology Information.

Southern Blot Analysis

Total DNA was prepared from hot pepper leaves according to the protocol of Doyle and Doyle (1990) with a minor modification. Thirteen μg samples of DNA were digested with *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I, respectively, subjected to electrophoresis in 1% agarose gel and then blotted onto a Hybond-N+ filter (Amersham). The total insert of *CaOMT1* was radioactively labeled with [α-^{32}P]dCTP (3000 Ci/mM, Amersham) by random priming (Promega) and used as a probe. The filter was prehybridized,

hybridized, and washed in the same method used in the screening of the cDNA library.

Northern Blot Analysis

Total RNA was extracted from pericarps and leaves of hot pepper, according to the guanidinium thiocyanate method (Sambrook *et al.*, 1989). Eleven μg of total RNA was run on 1% formaldehyde agarose gel, blotted onto a Hybond-N+ filter (Amersham), prehybridized at 55°C and hybridized at 55°C with ^{32}P-labeled probes of which templates were approximately a 500 bp fragment digested by *Bam*HI and a 430 bp fragment double-digested by *Xba*I and *Hind*III, which were prepared from *CaOMT1*. For washing the filter, the same method in the screening of the cDNA library was used except for the temperature (55°C).

RESULTS AND DISCUSSION

Isolation of Full Length cDNA Clone

Two degenerated primers, OMT1 and OMT2, of which sequences were based on conserved regions of known OMT's of tobacco (Jaeck *et al.*, 1993), poplar (Dumas *et al.*, 1992), alfalfa (Gowri *et al.*, 1991), and maize (Collazo *et al.*, 1992), were made and used for cloning of partial cDNA in the PCR. After electrophoresing the PCR product, we observed three bands of approximately 500 bp, 600 bp, and 1000 bp on the gel. Since the DNA of 600 bp showed a high similarity to the known OMT cDNA of poplar and tobacco, we used the pOMT600 as a probe for isolating a full length cDNA clone. Among the positive clones selected after the first and second screenings, three clones were thought to have the correct size, approximately 1.4 kb for the full length which was based on the sizes of the known OMT cDNA's-tobacco, 1431 bp; poplar, 1368 bp; cider tree, 1452 bp (Poeydomenge *et al.*, 1994); alfalfa, 1368 bp; aspen, 1503 bp (Bugos *et al.*, 1991). As results of sequence analyses and homology searches, one of them was identified as a full length OMT cDNA and designated *CaOMT1*.

Sequence Analyses of *CaOMT1* and CaOMT1

The cDNA of *CaOMT1* is 1377 bp in length, which consists of a 5'-leader sequence (58 bp), an open reading frame (1080 bp), and a 3'-untranslated region (239 bp) with a polyA tail (18 bp) (Fig. 1).

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   1                    TCCTATTTTTCTACTTCCCTTAGCTTGCCTAATTCAGTTATATCCAAAAATCTGAAAA    58

  59  ATG GAT TCA ACT AAC CAA AAC CTA ACT CAA ACA GAA GAT GAA GCA TTC TTA TTT GCA ATG   118
   1  Met Asp Ser Thr Asn Gln Asn Leu Thr Gln Thr Glu Asp Glu Ala Phe Leu Phe Ala Met    20

 119  CAA TTG GCT AGT GCT TCT GTA CTT CCT ATG GTC CTA AAA TCA GCT TTA GAA CTT GAC CTT   178
  21  Gln Leu Ala Ser Ala Ser Val Leu Pro Met Val Leu Lys Ser Ala Leu Glu Leu Asp Leu    40

 179  CTT GAA ATT ATG GCT AAG GCT GGT CCA GGT GCA GCC ATT TCT CCT TCT GAG CTT GCT GCT   238
  41  Leu Glu Ile Met Ala Lys Ala Gly Pro Gly Ala Ala Ile Ser Pro Ser Glu Leu Ala Ala    60

 239  CAG CTC CCT ACG AAG AAT CCA GAG GCA CCC GTT ATG CTT GAT CGA ATG CTT AGG CTT CTC   298
  61  Gln Leu Pro Thr Lys Asn Pro Glu Ala Pro Val Met Leu Asp Arg Met Leu Arg Leu Leu    80

 299  GCT ACT TAC TCT GTG CTT AAT TGT ACA CTC AGG ACC CTC CCT GAT GGC CGC GTA GAG CGG   358
  81  Ala Thr Tyr Ser Val Leu Asn Cys Thr Leu Arg Thr Leu Pro Asp Gly Arg Val Glu Arg   100

 359  CTT TAT AGT CTG GCT CCG GTG TGT AAG TTA CTG ACT AAG AAT GCA GAT GGT GTT TCT GTT   418
 101  Leu Tyr Ser Leu Ala Pro Val Cys Lys Leu Leu Thr Lys Asn Ala Asp Gly Val Ser Val   120

 419  GCC CCA CTT TTG CTC ATG AAT CAA GAT AAA GTC CTC ATG GAG AGC TGG TAC CAC TTA ACA   478
 121  Ala Pro Leu Leu Leu Met Asn Gln Asp Lys Val Leu Met Glu Ser Trp Tyr His Leu Thr   140

 479  GAT GCA GTA CTT GAT GGT GGA GTC CCA TTC AAC AAG GCT TAT GGA ATG ACA GCA TTC GAG   538
 141  Asp Ala Val Leu Asp Gly Gly Val Pro Phe Asn Lys Ala Tyr Gly Met Thr Ala Phe Glu   160

 539  TAC CAT GGT ACG GAT CCA AGA TTC AAC AAG GTT TTC AAC CGT GGA ATG TCT GAT CAC TCT   598
 161  Tyr His Gly Thr Asp Pro Arg Phe Asn Lys Val Phe Asn Arg Gly Met Ser Asp His Ser   180

 599  ACC ATG ACC ATG AAG AAG ATT CTA GAA GAC TAC AAA GGA TTT GAA GGA CTC AAT TCC ATT   658
 181  Thr Met Thr Met Lys Lys Ile Leu Glu Asp Tyr Lys Gly Phe Glu Gly Leu Asn Ser Ile   200

 659  GTT GAT GTT GGT GGT GGA ACT GGG GCT ACT GTG AAC ATG ATT GTC TCC AAG TAT CCG TCC   718
 201  Val Asp Val Gly Gly Gly Thr Gly Ala Thr Val Asn Met Ile Val Ser Lys Tyr Pro Ser   220

 719  ATT AAA GGC ATT AAC TTT GAT TTG TCA CAT GTT ATT GAA GAT GCT CCA GCT TAC CCC GGT   778
 221  Ile Lys Gly Ile Asn Phe Asp Leu Ser His Val Ile Glu Asp Ala Pro Ala Tyr Pro Gly   240

 779  GTC GAA CAT GTT GGT CGG GAC ATG TTT GTT AGT GTA CCG AAA GCA GAT GCC ATT TTC ATG   838
 241  Val Glu His Val Gly Arg Asp Met Phe Val Ser Val Pro Lys Ala Asp Ala Ile Phe Met   260

 839  AAG TGG ATT TGT CAT GAT TGG AGC GAT GAG CAT TGC TTA AAA TTC TTG AAG AAC TGT TAC   898
 261  Lys Trp Ile Cys His Asp Trp Ser Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr   280

 899  GAA GCA CTT CCT GCA AAT GGG AAA GTT TTA GTT GCA GAG TGC ATA CTT CCA GAG ACC CCA   958
 281  Glu Ala Leu Pro Ala Asn Gly Lys Val Leu Val Ala Glu Cys Ile Leu Pro Glu Thr Pro   300

 959  GAC ACA TCA GCT GCC ACA AAG AAT GCA GTA CAT GTT GAT ATT GTT ATG TTA GCA CAT AAC  1018
 301  Asp Thr Ser Ala Ala Thr Lys Asn Ala Val His Val Asp Ile Val Met Leu Ala His Asn   320

1019  CCA GGA GGC AAA GAA AGG ACT GAG AAA GAA TTT GAA GCT TTG GCT AAG GGT GCT GGA TTT  1078
 321  Pro Gly Gly Lys Glu Arg Thr Glu Lys Glu Phe Glu Ala Leu Ala Lys Gly Ala Gly Phe   340

1079  ACT GGA TTC CGC AGA GCT TGC TGT GCT TAC CAA ACT TGG GTC ATG GAA TTC CAC AAG TGA  1138
 341  Thr Gly Phe Arg Arg Ala Cys Cys Ala Tyr Gln Thr Trp Val Met Glu Phe His Lys ***   360

1139  ATTATCCAATTCCTTTGAAGGATTCAAAACAAAAATTGTTTACCCCTATATATATAGTGTTCATTTTGCAATTGGGATT  1217

1218  TTACTTTTACTCTGAGTGGCTTGACTGCGATGTAGTAGTGATCACTGTGAAATAAAAGAAAATTAGCTTTTACATTG  1294

1295  ATGAGATTTATGTTCACTATGAAATAATTGCTCTTAGAAAAAGAATGTGTTCAATATAAAGTTCAAAAAAAAAAAAAAAAAA  1377
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Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the *CaOMT1* cDNA clone. The terminal codon is indicated by asterisks (***). Residues homologous to the conserved flanking sequence of ATG are underlined. Putative glycosylation sites and polyadenylation signals are shadowed and boldfaced, respectively. The GenBank accession number of this sequence is U83789.

Its [G+C] content is 40.5%.

In the 58 bp of the 5'-leader sequence, [A+T] content is 69%, which corresponds to analyses of 79 plant genes, in which 53% of the plant genes had 40~50 bp of the 5'-leader sequence and 92% of them had over 50% of [A+T] content in them (Joshi, 1987a). The translation start region shows a high similarity of 87.5% to the plant consensus initiation context, AACAATGG and corresponded to purine at 3 bp upstream of ATG (Guerineau, 1992). Moreover,

it corresponded to the report that at 3 bp upstream of ATG in dicots adenine appears 2~4 times more frequently than in monocots (Cavener *et al.*, 1991). In the 3'-untranslated region, there are three sequences (CACTG, AATAAA, CATTG) which seem to be polyadenylation signals and are located at 100 bp, 92 bp, and 70 bp upward from polyA tail, respectively (Joshi, 1987b).

The 1080 bp open reading frame encodes a polypeptide of 359 amino acids, whose calculated molecular weight is 39,430 D, similar to that of the known OMT's - *e.g.* 38.5 kD~43 kD of tobacco (Hermann *et al.*, 1987); 38 kD of poplar (Doorsselaere *et al.*, 1993); 40 kD of aspen (Bugos *et al.*, 1991); 41 kD of alfalfa (Vance and Bryan, 1981); 42±2 kD of cabbage (De Carolis and Ibrahim, 1989). It also has five conserved boxes, which appear in all known OMT's, consisting of *S*-adenosyl-L-methionine binding motifs (Boxes I, III, and IV) (Ingrosso *et al.*, 1989), Box I-extended, conserved motif (Box II), and conserved hydrophilic motif (Box V) (Fig. 2).

As a result of the NCBI BLAST sequence similarity search, the nucleotide sequence of *CaOMT1* had 89~74% identity with the OMT cDNA of tobacco, aspen, alfalfa, and poplar, but a lower identity of 59% with the OMT cDNA of maize, a monocot. Amino acid sequence analysis also revealed that CaOMT1 has high identity with the known OMT's which have a substrate of *o*-diphenolic compounds, especially 5-hydroxyferulic acid and caffeic acid. This supports the idea that *CaOMT1* encodes an OMT. In order to confirm the activity of CaOMT1, we are trying to express CaOMT1.

Based on the result of the NCBI Entrez search, CaOMT1 is the first reported cDNA clone for enzymes related to the phenylpropanoid pathway in hot pepper. We think *CaOMT1* will be helpful to study not only as regards capsaicin biosythesis and its regulation, but also in phenylpropanoid pathway related phenomena-pathogenic response (Jaeck *et al.*, 1993), stress response (Gowri *et al.*, 1991), and lignin biosynthesis (Grisebach, 1981).

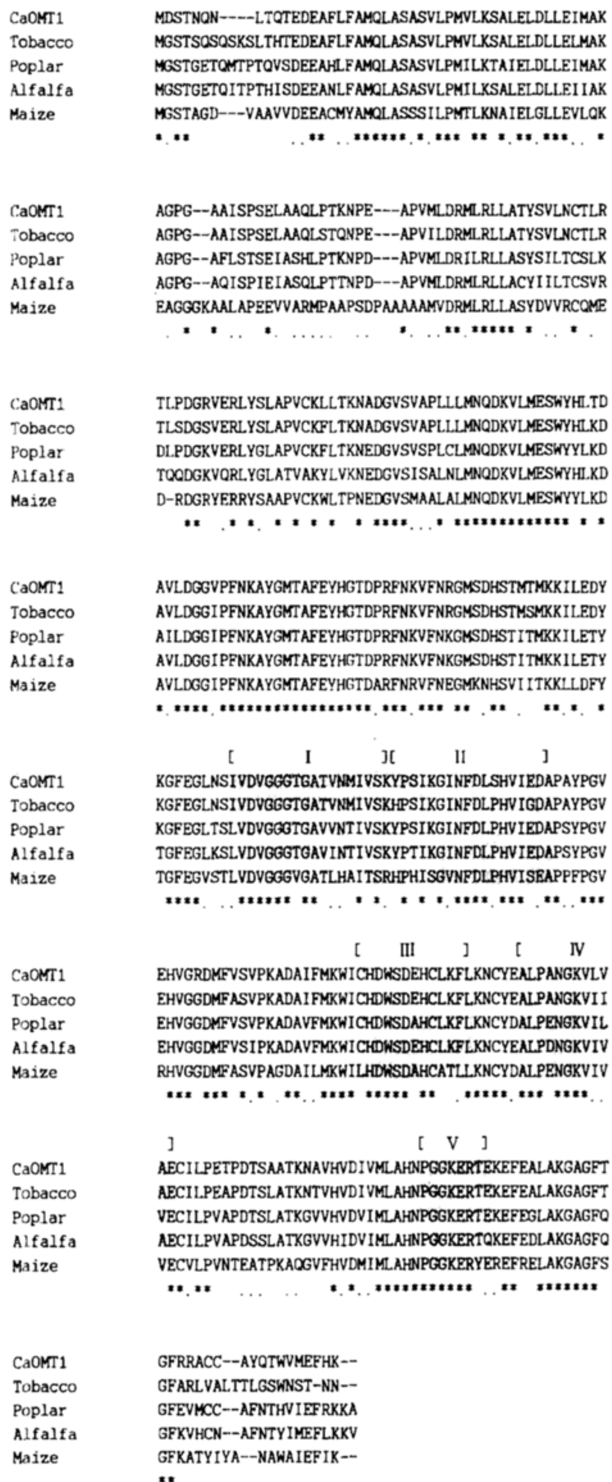
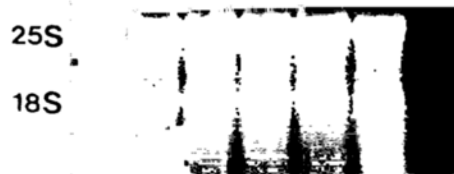


Fig. 2. Alignment of the deduced amino acid sequences of CaOMT1 and OMT of tobacco, poplar, alfalfa, and maize. The amino acids identical in four proteins are marked with asterisks (*) and the amino acids similar in four proteins are marked with dots (·). The five conserved boxes in OMT are shadowed.

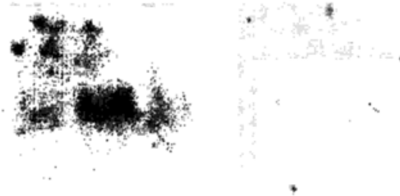
Southern Blot Analysis and Northern Blot Analysis

We could observe several hybridizing signals in the Southern gel blot (Fig. 3). It suggested that the *CaOMT1* gene might exist in the form of multiple copies in the hot pepper genome. However, the overall intensity of the hybridizing signals was not even suggesting that besides the genomic sequence

Fig. 3. DNA gel blot analysis of the gene for CaOMT1 in *Capsicum annuum*. Each 13 μg of *Capsicum annuum* total DNA digested with *Eco*RI (EI), *Eco*RV (EV), *Hind*III (H), and *Xba*I (X) was separated on a 1.0% agarose gel, transferred to a Hybond-N+ filter and probed with the *CaOMT1* cDNA clone.



(a)



(b)

Fig. 4. RNA gel blot analysis of total RNA from hot pepper pericarp. Each 11 μg of total RNA extracted from the pericarp of young stage (Y), fully developed adult stage (A), red-turning stage (T), fully ripened stage (R), and a leaf (L) was separated on a 1.0% formaldehyde agarose gel. (a), ethidium bromide staining; (b), autoradiogram hybridized with radiolabeled *CaOMT1*.

of *CaOMT1* itself, other hybridizing sequences were distantly related. Jaeck *et al.* (1993) showed that the tobacco genome has two OMT genes as a result of Southern blot. Besides, it was reported that there are two or three OMT genes in the genomes of alfalfa (Gowri *et al.*, 1991) and aspen (Bugos *et al.*, 1991). Therefore our result agrees with these previous studies.

In order to study the *CaOMT1* mRNA level during fruit growth, the fruit growth period was divided into four stages based on the length and color of the fruit (Hubbard and Pharr, 1992; Ochoa-Alejo and G mez-Peralta, 1993) - young stage, fully developed stage, red-turning stage, and fully ripened stage.

The two former stages were considered as developmental, and the latter two as ripening. Total RNA was extracted from the pericarp of each stage and a leaf. The result of Northern blot analysis showed that *CaOMT1* mRNA expression level increased during pepper fruit development, but decreased during ripening (Fig. 4). The result agrees with the report that the activity of OMT in pepper increases during development and decreases 30 days after flowering (Ochoa-Alejo and Gómez-Peralta, 1993). This means the *CaOMT1* gene is fruit development-related, not ripening-related like geranylgeranyl pyrophosphate synthase (Kuntz *et al.*, 1992), cysteine synthase (*O*-acetylserine sulfhydrylase) (Romer *et al.*, 1992), or ascorbate peroxidase (Schantz *et al.*, 1995) which are known to increase their activities and expressions during ripening of pepper. It was also observed that *CaOMT1* mRNA was not detected in the total RNA of hot pepper leaf suggesting that *CaOMT1* is expressed preferentially in fruit organ. The facts that callus derived from the hypocotyl of pepper had low OMT activity (Ochoa-Alejo and Gómez-Peralta, 1993), that xylems of poplar (Doorsselaere *et al.*, 1993), aspen (Bugos *et al.*, 1991), and tobacco (Jaeck *et al.*, 1992) had tissue-specifically high OMT mRNA levels, and that high OMT mRNA levels were detected in roots and stems of alfalfa (Gowri *et al.*, 1991) support our speculation.

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